

1      **Ubiquity of polystyrene digestion and biodegradation within yellow mealworms,**  
 2                      **larvae of *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae)**

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## ABSTRACT

Academics researchers and “citizen scientists” from 22 countries confirmed that yellow mealworms, the larvae of *Tenebrio molitor* Linnaeus, can survive by eating polystyrene (PS) foam. More detailed assessment of this capability was carried out for mealworms from 12 sources: five from the USA, six from China, and one from Northern Ireland. All of these mealworms digested PS foam. PS mass decreased and depolymerization was observed, with appearance of lower molecular weight residuals and functional groups indicative of oxidative transformations in extracts from the frass (insect excrement). Addition of gentamycin (30 mg g<sup>-1</sup>), a bactericidal antibiotic, inhibited depolymerization, implicating the gut microbiome in the biodegradation process. Microbial community



analyses demonstrated significant taxonomic shifts for mealworms fed diets of PS plus bran and PS alone. The results indicate that mealworms from diverse locations eat and metabolize PS and support the hypothesis that this capacity is independent of the geographic origin of the mealworms and likely ubiquitous to members of this species.

**Keywords:** Plastic wastes, Polystyrene, Degradation, Mealworms, *Tenebrio molitor*, gut microbiome

## 1. Introduction

The accumulation of petroleum-based synthetic plastic wastes, including polystyrene (PS), polyethylene (PE), polypropylene (PP), polyurethane (PUR), polyvinyl chloride (PVC), and polyethylene terephthalate (PET), is a major environmental concern, with up to 6,300 million metric tons generated to date (Geyer et al., 2017). Plastic degradation is defined as a physical or chemical change in the polymer due to environmental factors, such as light, heat, moisture, chemical conditions or biological activity (Shah et al., 2008). During microbial biodegradation, polymers are first depolymerized to smaller monomers which are further degraded for energy or assimilated. Ultimate biodegradation or mineralization is achieved when the degraded monomers are converted to CO<sub>2</sub>, water, and stabilized microbial biomass. Most petroleum-based plastics are resistant to biodegradation (Otake et al, 1995, Shah et al, 2008). One of the most commonly used plastics is PS,  $[-CH-(C_6H_5)CH_2-]_n$ , constituting 7.1% of annual plastic production (Wu et al., 2017). PS products and materials are used in diverse applications, including eyeglasses frames, plastic cups, egg trays, packaging, and building insulation (Plastics

Europe, 2016). As a consequence, PS has become a major pollutant of soils, rivers, lakes, and oceans (Zhou et al., 2011) and a source of microplastics (<5 mm) (Hidalgo-Ruz et al, 2012; Wu et al., 2017). Although resistant to microbial biodegradation in a wide range of habitats (Guillet et al., 1974; Jones et al., 1974; Sielicki et al., 1978; Kaplan et al., 1979; Mor and Sivan, 2008; Råberg et al., 2008), PS is now known to be susceptible to rapid biodegradation in the gut of yellow mealworms (Yang et al., 2015a, 2015b; Yang et al. 2018; Brandon et al. 2018), the larval form of *Tenebrio molitor* Linnaeus (Howard, 1955; Roberson, 2005; Löbl et al., 2008).

*T. molitor* belongs to the order Coleoptera (beetles) and family Tenebrionidae (common name “darkling beetle”), a cosmopolitan family of beetles with more than 20,000 species. Since the 1950’s, researchers have observed that some beetles and larvae in the Tenebrionidae family damaged plastic packaging materials (Gerhardt and Lindgren, 1954; Cline, 1978; Newton, 1988). Subsequently, consumption of PS foams by mealworms was reported by students competing in high school science fairs: in 2003, Ms. Chong-Guan Chen raised mealworms fed PS foam and theorized that the PS biodegraded (Sina, 2003); in 2009, Ms. I-Ching Tseng isolated bacteria from mealworms gut using PS as the sole carbon source (Burkart, 2009). Convincing academic evidence of PS degradation was reported in mealworms from Beijing, China (Yang et al., 2015a) then in a mealworm strain from a pet store in the United States (Yang et al., 2018). After a 1-2 week adaptation period, the polymer fraction of ingested Styrofoam decreased by up to 65% with a 12-15 hr gut retention time (Yang et al., 2018). Almost half of the ingested PS carbon was converted into CO<sub>2</sub> in the mealworm gut (Yang et al. 2015a; Brandon et al., 2018). Isotopic studies using <sup>13</sup>C-labeled PS materials have shown that PS was

mineralized to  $^{13}\text{CO}_2$  and incorporated into lipids (Yang et al., 2015a). After being fed with the antibiotic gentamycin, mealworm strain from Beijing almost completely lost the ability to depolymerize PS and mineralize PS into  $\text{CO}_2$ , implicating gut bacteria in PS biodegradation (Yang et al., 2015b). Additional studies on the same strains of mealworms isolated a PS-degrading bacteria strain (*Exiguobacterium sp.*) (Yang et al. 2015b) and indicated that co-feeding with bran resulted in enhanced PS degradation and enabled mealworms to consume PS for multiple generations (Yang et al. 2018).

In this study, we test the hypothesis that PS degradation in mealworms is ubiquitous. Evaluation of this hypothesis is needed because prior work has focused on mealworms from just two sources (Yang et al., 2015a; Yang et al., 2018). To assess plastic biodegradation for mealworms from a broader range of sources, we enlisted the help of academic and non-academic institutions from around the world to collect information about mealworm behavior related to plastic consumption. We also obtained mealworms from 12 different sources and tested them for PS degradation using standard protocols (Yang et al., 2015a; Yang et al., 2018), including PS mass balances to determine the specific rates of PS degradation; (2) gel permeation chromatography (GPC) to assess changes in molecular weight, and (3) Fourier transform infrared (FTIR) spectroscopy of frass residues to identify chemical modifications resulting from PS digestion.

We find that mealworms from diverse locations share the ability to consume and degrade PS. We also report observation that add to the existing body of evidence that the gut microbiome plays a role in PS depolymerization and degradation. These microbiomes differ for microorganisms from different sources, with shifts in microbial community structure observed when mealworms are fed PS.

## 2. Methods

### 2.1. Worldwide survey

One of the co-authors (W.-M. Wu, Stanford University) organized and supervised a worldwide survey with volunteer investigators from around world, including middle school and high school students, university students, school teachers, professors, mealworm farmers and mealworm breeders. These diverse collaborators obtained locally available mealworms and fed them PS materials, including Styrofoam, XPS, and other PS foams. Information of the location of the investigator, source of mealworms, test materials and plastic-eating and consumption behavior is summarized in the Supplementary data. Web searches for literature, science fair news and YouTube videos were also used to obtain information on mealworms eating patterns and degradation of PS and other plastics within mealworms.

### 2.2 Source of mealworms

Mealworms (developmental stage: instar 5 -7) from the twelve sources (five in the USA, six in China, and one in the United Kingdom) were obtained by: (1) direct purchase from the PetCo Pet Store Chain, Mountain View, California (supplied to the store by Timberline Fisheries); (2) direct purchase from the PetSmart Pet Store Chain, Sunnyvale, California (supplied by Bug Company, Ham Lake, Minnesota ); (3) mail order from Timberline Fisheries, Marion, Illinois (ordered separately from source 1); (4) mail order from Exotic Nutrition Pet Company, Newport News, Virginia; (5) mail order from Rainbow Mealworms, Compton, California; (6) direct purchase from a pet store in Belfast, Northern Ireland, UK; (7) direct purchase from a pet store in Beijing; (8) mail order from a store in Harbin, Heilongjiang Province; (9) direct purchase from a mealworm farm in Tai'an County, Shandong Province; (10) direct purchase from a pet store in Xi'an City, Shaanxi Province; (11)

direct purchase from a pet store in Shanghai; (12) mail order from a pet store in Shenzhen City, Guangdong Province (Table 1). All larvae were identified as *Tenebrio molitor* Linnaeus based on morphology and coloration. Prior to arrival, and for at least two days prior to PS degradation tests, the larvae were fed bran and maintained in incubators with bran.

### 2.3. PS feedstocks

Mealworms received one of three PS feedstocks, labeled A, B, or C. These feedstocks had similar density ( $0.020\text{--}0.021\text{ g cm}^{-3}$ ) and similar molecular weights (Table S1, Supplementary data). Average molecular weights of the feedstock materials were determined in the analytical laboratories of the Department of Chemistry, Stanford University. Feedstock A consisted of Styrofoam plates from Insulfoam (Carlisle Construction Materials, Puyallup, WA), the number-average molecular weight ( $M_n$ ) was  $90,000 \pm 2,000$ , and the weight-averaged molecular weight ( $M_w$ ) was  $228,000 \pm 1,000$ . Feedstock B consisted of white EPS70 SDN insulation Styrofoam from UK Packaging Suppliers (Staffordshire, UK), with  $M_n$  of  $89,000 \pm 2,000$ , and  $M_w$  of  $210,000 \pm 2,000$ . Feedstock C consisted of Styrofoam packing material from Shenzhen, China, with  $M_n$  of  $93,000 \pm 3,000$ , and  $M_w$  of  $254,000 \pm 1,000$ .

### 2.4. PS biodegradation capability

Mealworms in developmental stages instar 7-8 were cultivated in food grade high density polypropylene (PP) storage containers in laboratories at Stanford, CA, USA; Beijing, China; and Belfast, UK. The set up and methods used were similar to those previously reported (Yang et al., 2018). At Stanford, 120 mealworms were incubated in 475 mL PP containers; in Northern Ireland 200 mealworms were incubated in 600 mL containers; in China, 500 mealworms were incubated in 1200 mL containers. In each



incubator, the spatial density of the mealworms was  $\sim 2$  worms  $\text{cm}^{-2}$ . The humidity within the containers was maintained at 75-80%. Temperature was maintained at 25 °C at Stanford, 24-26 °C in Belfast, and 26-27 °C in Beijing. Mealworms from US sources 1-5 and Chinese sources 7-11 received Feedstock A; mealworms from the UK source 6 received Feedstock B; mealworms from Chinese source 12 received feedstock C. All tests in the USA and UK were performed in duplicate. An additional test was performed at Tongji University: mealworms from source 11 (Shanghai) were tested for survival under different feed conditions at ambient temperature (25-26 °C) over a 34-day test period.

At Stanford, mealworms were fed PS alone and PS plus bran. To initiate these tests, 1.80 g PS feedstock was added to each incubator. For the PS alone diet, no additional PS was added thereafter. For the PS plus bran diet, each incubator received 3.6 g bran supplements every 4 days, resulting in a cumulative bran to PS ratio (B/PS) of 14:1 over a 32-day period (Yang et al., 2018). In Belfast, all incubators initially received PS (1.0 g). For the PS alone diet, no additional PS was added thereafter. For the PS plus bran diet, bran supplements (1.0 g) were provided weekly. Unfed mealworms were used as control. In Beijing, mealworm incubators were fed PS plus bran. Each incubator initially received 8.3 g PS plus 14.8 g bran; this was followed by 14.8 g bran supplements every 4 days thereafter, resulting in a cumulative B/P ratio of 8.9:1 over a 24-day period. In tests at all three labs, dead mealworms were removed whenever observed. Tests conducted at Tongji University were carried out in four incubators initially containing 410 mealworms per incubator. Mealworms were fed one of three diets at the beginning : (1) 8.5 g bran, (2) 4.70 g PS, and (3) 4.7 g PS plus 8.5 g bran. Bran was supplemented every 5 days with a cumulative B/P ratio of 14.5:1. Unfed controls were also evaluated.

Mealworm survival rates (SRs) and PS mass losses were monitored and calculated. For DNA analyses, mealworms samples (at least 30) were collected, preserved with 100% ethanol (analytical grade) and stored at -80 °C.

## 2.5. Characterization of feedstock and frass

Biodegradation and depolymerization of ingested PS was assessed by GPC and FTIR analyses of feedstock and frass. Frass from each incubator was collected and extracted as described in the Supplementary data and a previous report (Yang et al., 2018). The respective feedstock PS materials were extracted using the same procedures as frass samples prior to GPC analysis. Triplicate GPC analyses were carried out at Stanford University to determine  $M_n$ ,  $M_w$  and molecular weight distribution (MWD) of feedstock and frass samples. Triplicate 100- $\mu$ L frass extract samples were injected into a GPC operating at a THF eluent flow rate of 1.0 mL min<sup>-1</sup> at 40 °C (Viscotek GPCmax VE 2001 GPC Solvent/Sample Module, Viscotek Corporation, Houston, Texas, USA). Triplicate FTIR analyses (EQUINOX 55 FT-IR Spectrometer, Bruker Corporation, Ettlingen, Germany) were performed at Tongji University to characterize functional groups in PS feedstocks and frass samples over the range 400-4000 cm<sup>-1</sup>.

## 2.6. Effect of Antibiotics on PS Degradation

The effect of antibiotic suppression on PS depolymerization and degradation was tested using mealworms from US sources 1-5 and gentamicin as an inhibitor. Gentamicin was selected based on the results of Yang et al. (2015b). Mealworms (120 per container) were fed bran mixed with gentamicin sulfate (Cat# 00149, Lot# 001204-15032306, Chem-Impex Int'l Inc., USA) at a dosage of 30 mg gentamicin sulfate per gram of bran. Controls were fed bran alone. On day 0, 7, and 15, 15 mealworms in each incubator were

randomly chosen from each source of mealworms to prepare a gut suspension for counting of gut microorganisms. The surfaces of the worms were decontaminated by immersion in 70% ethanol for 1 min then rinsing twice with distilled water. The gut contents of 15 mealworms from each source were then extracted and suspended in 5 ml sterilized saline water (7.5% NaCl). This initial gut suspension was diluted using a five-step ten-fold serial dilution ( $10^{-1}$  to  $10^{-5}$ ) and spread on Trypticase soy agar (TSA) plates containing 15 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> soytone, 5 g L<sup>-1</sup> sodium chloride, 15 g L<sup>-1</sup> agar, pH 7.3± 0.2, a general-purpose, nonselective medium providing enough nutrients to allow for a growth of a wide variety of microorganisms. After incubation for 24 hrs (at 30 °C), the number of colonies were counted. On day 7, mealworms from the antibiotic treatment and the control treatment were fed PS feedstock. Gentamicin sulfate mixed bran (30 mg gentamicin sulfate g bran<sup>-1</sup>) was then continuously supplied to the treatment group until day 15. Over the 15-day incubation period, frass samples from both treatment and control incubations were collected for GPC analysis.

## 2.7. Gut Microbial community analysis

Mealworm samples from each strain were collected, preserved in 100% ethanol at -80 °C, and shipped to the lab for microbial community analysis. DNA extraction from externally decontaminated larval mealworms was performed using the DNeasy Blood and Tissue kit (Qiagen) before library construction and sequencing (2 x 250 bp paired-end reads, Illumina MiSeq) were performed at the Michigan State University Genomics Core Facility. Sequencing reads were filtered and taxonomically assigned using QIIME 2. Additional methods are available in the supplemental materials.

### 3. Results and discussion

#### 3.1. Ubiquity of plastic eating behavior among mealworms

Survey responses from collaborators in 22 countries (Fig. 1) indicated that mealworms in all locations consumed PS foam, including mealworms from North America (Canada, Mexico, USA); Asia (Cambodia, China, Japan, Indonesia, Iran, Israel, South Korea, Thailand); Europe (Finland, France, Germany, Poland, Slovenia, Spain, Turkey, UK); Africa (Nigeria, South Africa) and Australia. From these results, we surmise that chewing and ingestion of PS is likely an adaptive behavior intrinsic to yellow mealworms, which feed upon decaying forest vegetation in natural settings (Howard, 1955). Marshall School students (Duluth MN, USA) reported 33 mg PS 100 worms<sup>-1</sup> d<sup>-1</sup>, within the range of previous tests; a team at the University of Queensland, St. Lucia, Australia, reported that local mealworms consumed PS at a rate of 13 mg PS 100 worms<sup>-1</sup> d<sup>-1</sup>; a team in Bandar Lampung, Indonesia, reported that PS foam feeds supported mealworm growth, and the feed had no significant effect on larvae mortality (Nukmal et al., 2018); and students reported formation of beetles from larvae when fed PS plus bran, confirming prior reports (Yang et al., 2015a; Yang et al., 2018). Mealworms were also observed consuming PE, an observation now documented in the peer-reviewed literature (Brandon et al., 2018), as well as other plastics, including PVC and PLA, as reported by an academic team in Poland (Bożek et al., 2017).

#### 3.2. Ubiquity of biodegradation of PS

The ubiquity of PS biodegradation was evaluated by testing mealworms from twelve distinct geographic locations with three PS feedstocks (Table 1). All mealworms chewed and ingested PS (Fig. 2). For mealworms from sources 1-6, 32-day survival rates (SRs)

for mealworms fed PS alone exceeded 80% (Table 1). These values were statistically indistinguishable from the 32-day SRs of mealworms fed PS plus bran (Table 1).

Over a 32-day period, SRs for mealworms from US sources 2 through 5 were higher for mealworms fed bran only (88-90%), PS plus bran (85-92%), and PS only (83-92%) than for unfed mealworms (69-76%) (Table 1 and Fig. S2, Table S1). Higher SRs were also observed in mealworms from source 11 (Shanghai) when fed PS plus bran (95%) or PS only (92%) compared to unfed mealworms (68%) over a 34-day period (Fig. S1C, Table S2).

The average weight of mealworms from source 6 (Northern Ireland) increased by 22% over a 34-day period when fed bran or PS plus bran, but only increased by 7.3% when fed PS alone, and decreased by 8.2% when unfed (Table 1). Similarly, the average weight of mealworms from source 11 (Shanghai) increased by 16-25% over a 34-day period for mealworms fed bran or PS plus bran, but decreased by 9.3% when fed PS alone and by 17.4% when unfed (Table S2, Supplementary data).

These results indicate that digestion of ingested PS can provide significant energy (source 6) and offset energy requirements for maintenance for over one month (source 11). In a previous test of the Beijing strain fed  $\alpha$   $^{13}\text{C}$ - and  $\beta$   $^{13}\text{C}$ -labelled PS (Yang et al., 2015a), individual fatty acids had  $\delta$   $^{13}\text{C}$  values that were significantly higher than bran-fed controls, especially in unsaturated FAs, indicating that  $^{13}\text{C}$  from the  $^{13}\text{C}$ -labeled PS was assimilated into mealworm biomass. This is evidence that PS digestion can provide both energy and carbon for mealworm maintenance and survival during the first month of cultivation (Yang et al., 2015a; Yang et al., 2018). Further studies are needed to understand the conditions regulating the mechanisms of energy recovery from

plastic consumption. Whether the mealworms obtain sufficient energy for maintenance likely depends upon the PS consumption rate and digestibility of the PS consumed.

In the 98-day tests of mealworms from source 6, the SRs of mealworms fed PS alone matched those fed PS plus bran during the initial 35 days of incubation (95.5% versus 98.0%) then dropped to low levels, like those of unfed controls (Table 1). Further investigation revealed that both the unfed mealworms and mealworms fed PS alone engaged in cannibalism. The 98-day SR was  $11.8 \pm 1.1\%$  for unfed mealworms, and  $11.5 \pm 4.9\%$  for mealworms fed PS alone. By contrast, the 98-day SR for mealworms fed bran plus PS was  $81.5 \pm 7.1\%$  (Fig. S1B). Because PS contains only hydrogen and carbon, it does not provide adequate nutrition (N, P, Na, K, trace elements, amino acids, etc.) for long-term survival and growth. The positive effect of PS on SR does not last for long time due to the lack of nitrogen sources and other nutrients. Addition of bran relieved this constraint. In the absence of added bran, however, PS-fed mealworms survived by consuming dead mealworms and their molts. The survivors (about 12% of the original population) maintained a biomass similar to those that received bran (Table 1).

Specific PS consumption rates from US sources 1-6 and Northern Ireland UK source 7 were compared for mealworms fed PS alone and bran plus PS (Table 1). Mealworms fed bran plus PS typically consumed PS at rates that were more than twice those of mealworms fed PS alone. Not surprisingly, mealworms from sources 1 and 2 (same source, but different batch) had similar PS specific consumption rates. The initial size of the mealworms had no clear impact on the rate of PS consumption, but the lightest

mealworms (source 6; average weight 33.5 mg) consumed PS at the highest rate (7.64 mg PS g worms<sup>-1</sup> d<sup>-1</sup>).

For the mealworms obtained in China, PS consumption rates were evaluated under relatively optimal conditions. While the results varied, the ranges observed were similar to those of mealworms from the USA and Northern Ireland (Table 1).

### 3.3. Characterizations of the egested frass

During incubation with PS as a feedstock, decreases in  $M_n$  and  $M_w$  and MWD shifts to were monitored (Albertsson et al., 1998; Yang et al., 2015a; Yang et al., 2018). Fig. 3 shows evidence of PS depolymerization within the guts of mealworms from all 12 sources, with significant decreases in  $M_n$  and  $M_w$  accompanied by shifts in MWD to lower molecular weights (t test  $p < 0.05$  in Table S2).

Frass samples from mealworms from US sources 1-5 and Chinese sources 7-11 contained polymer extracts with  $M_n$  values that were 9.1-15.6% lower than the feedstock and  $M_w$  values that were 5.2 -10.8% lower than the feedstock (Feedstock A with  $M_n$  of 90,000±2000;  $M_w$  of 228,000±1,200) (Table S3, Supplementary data). Frass samples from mealworms from Northern Ireland (source 6) had  $M_n$  values that were 5.4 % lower and  $M_w$  values that were 12.1% lower than the feed (Feedstock B with  $M_n$  of 89,000±2,000;  $M_w$  of 210,000±2,000). For frass samples from Chinese source 12,  $M_n$  values were 11.2 % lower, and  $M_w$  values were 9.0% lower than the feed (Feedstock C with  $M_n$  of 93,000 ± 3,000 and  $M_w$  of 250,000±1000 (Table S3). These decreases in  $M_n$  and  $M_w$  were significant for all sources (t-test,  $p < 0.05$ , Table S2), indicating depolymerization and degradation of PS feedstock was ubiquitous across all 12 source populations.

FTIR analyses verified the formation of new functional groups during passage through the mealworm gut. Comparison of FTIR spectra for the PS feed stock (control) and egested frass of all tested mealworms showed chemical changes and incorporation of oxygen previously linked to plastic degradation (Fig. 4 A and 4B, Fig. S3 and S4, Supplementary data). These data are similar to those previously reported (Yang et al., 2018, Brandon et al., 2018). The spectra of frass from mealworms fed with PS and PS plus bran are significantly different from the frass of mealworms fed bran only (Fig. 4A and Fig. S3, Supplementary data). Peaks at 625-970  $\text{cm}^{-1}$  (ring-bending vibration) had high intensities in the PS feedstock but were much weaker in frass samples. Peaks characteristic of the PS benzene ring (C=C stretch, 1550-1610 and 1800-2000  $\text{cm}^{-1}$ ) were dampened in frass of mealworms fed PS or PS plus bran, suggesting ring cleavage. The PS fed samples showed a decrease intensity for peaks characteristic of PS (Sekhar et al., 2016) and appearance of more carbonyl groups (C=O stretching, 1700  $\text{cm}^{-1}$ ) compared with samples fed bran alone or PS feedstock (Fig. 4A and 4B, Fig. S3, Supplementary data). The broadening of peaks at 2500-3500  $\text{cm}^{-1}$  in all FTIR spectra is associated with the hydrogen bond of hydroxyl groups and/or carboxylic acid groups, suggested a shift from hydrophobic to more hydrophilic surface properties. FTIR analysis confirmed that all frass samples showed strong signs of PS biodegradation, as observed in previous tests of mealworms from Beijing (Yang et al., 2015a) and a California PetCo store (Yang et al., 2018).

#### 3.4. Effect of gentamicin on PS depolymerization

Antibiotics, such as nystatin and ampicillin, suppress gut microbiota in mealworms and provide insight into the role that gut microbiota play in digestive processes, such as the



digestion of cell walls and glucoside detoxification (Genta et al., 2006). Previous studies have inferred that gut microbes were required for PS biodegradation due to suppression of PS degradation when gentamycin was added (Yang et al., 2015b). Gentamicin addition to the diets of mealworms from the five USA sources were used to examine the role of gut bacteria on PS depolymerization/biodegradation. After feeding gentamicin sulfate plus bran for 7 days, gut bacteria numbers dropped significantly from  $10^6$  levels by 2-3 magnitudes compared to controls (Fig. 4C and Table S5), indicating suppression of the gut bacteria. After 15 days, frass samples were collected for tetrahydrofuran (THF) extraction and GPC analysis of residual polymers. GPC analyses indicated inhibition of depolymerization when the mealworms were fed gentamicin-containing bran, but depolymerization remained elevated in the control treatment (Fig. 4D and 4E). No statistically significant differences were observed in  $M_w$  and  $M_n$  values between PS feed stock and residual polymers extracted from frass samples of mealworms receiving the gentamicin treatment for all five US sources (Table S6). On the other hand, significant differences were observed in  $M_w$  and  $M_n$  values between PS fed stock and residual polymers extracted from control and gentamicin treatments (Tables S6 and S7). This is evidence that gentamycin suppressed gut microbiota and inhibited PS depolymerization. From these results, we hypothesize that the depolymerization of PS may be induced by gut microbes that are directly in contact with PS or entail synergetic effects involving gut microflora and mealworm digestive enzymes within the gut. Our results showed that unfed mealworms showed slight but reproducibly lower SR. It seems that microbes in the gut of mealworms cause a partial depolymerisation of PS with the release of broken down substances which sustain the SR of mealworms even in the absence of other nutrients

(such as bran). Further study is needed to examine whether antibiotics causes lower SR for mealworms fed PS only.

### 3.5. Gut Microbial community analyses

The dominant bacterial phyla across all mealworm samples, representing greater than 99% average relative abundance in the measured bacterial communities, were Actinobacteria, Firmicutes, Proteobacteria and Tenericutes. The six most abundant families (> 98% total relative abundance on average) were *Bacillaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* representing a diverse range of mostly aerobic and/or facultative bacteria. Fig. 5 illustrates differences in bacterial taxa between mealworms fed normal feed bran (Original) and mealworms fed PS alone and PS plus bran (PS/Bran). The original bacterial communities varied greatly between source populations, likely due to differences in diet at different geographic locations and strain-specific properties (genes, growth condition and history). However, after mealworms switched diets from bran to PS plus bran or to PS alone, the gut microbial communities converged on similar patterns independent of the mealworm source.

At the phylum level, the relative abundance of Firmicutes decreased significantly in the PS-only diets ( $P = 0.0055$ ) and the PS plus bran diet (Wilcox,  $P = 0.0035$ ). There were no significant differences between the PS-only diet and the PS plus bran diet ( $P = 0.293$ ). Firmicutes include Clostridia (anaerobic bacteria) and Bacilli (aerobic bacteria), and both are spore formers. No other phyla were significantly different between treatments though Proteobacteria abundance showed an inverse relationship with Firmicutes (Fig. 5A). At the family level, *Enterobacteriaceae* represented 33.7% ( $SE \pm 6.3\%$ ) of total relative

abundance in the original diet,  $64.6 \pm 12.5\%$  in the PS-only diet, and  $50.2 \pm 6.5\%$  in the PS plus bran diet (Fig. 5B). After adjusting P values for multiple comparisons, there were no significant differences in relative abundance of any family. At the genus level, *Erwinia* was the most prevalent genus, representing  $25.3 \pm 5.65\%$  in the original treatment,  $49.1 \pm 17.6\%$  in the PS-only treatment, and  $31.2 \pm 5.64\%$  in the PS plus bran treatment, with no significant differences between diets (Fig. 5C).

When the diet of the mealworms shifted from bran to PS-only or PS plus bran, the gut microbiota shifted to a community with improved capabilities for PS degradation. At present, however, it is difficult to prove which microbial genera or families are responsible for enhanced PS degradation because only a few PS-degrading bacteria have been isolated. These include PS-degrading *Exiguobacterium* sp. YT2 (Yang et al., 2015b), Phylum: Firmicutes; Class Bacilli, and *Rhodococcus ruber* (Mor and Siva, 2008), Phylum Actinobacteria, Family *Nocardiaceae*. Recently, Tang et al., (2017) reported two bacterial strains isolated from the guts of mealworms and superworms' that are capable of growing on PS - *Aeromonas* sp and *Klebsiella pneumoniae* - both of which belong to the family *Enterobacteriaceae* (P *Proteobacteria*, C *Gammaproteobacteria*). It is reasonable to hypothesize that PS-degrading bacteria are able to secrete extracellular oxidative enzyme(s) that break down PS polymer chains and generate intermediates with C=O bonds; features present in broad families of aerobic or facultative bacteria. Additional isolation and characterization of PS-degrading species is needed to enable identification of key functional enzymes involved in the depolymerization and biodegradation of PS, PE and other plastics. Given the shifts in taxonomic composition that occurred after the mealworms were fed a diet containing PS, this work suggests that bacteria responsible for

depolymerizing PS and degradation of intermediates likely include Gram-positive Clostridia (anaerobic conditions), Gram-negative Enterobacteriaceae (anaerobic-aerobic conditions) and Gram-positive Bacillus (aerobic conditions).

The results of this study indicate that microbiomes capable of depolymerizing PS are widely present in mealworm gut from various sources and that shifts in gut microbial communities may be a direct result of PS consumption (i.e. a changing community to better degrade plastics) or an indirect response (i.e. a response to nutrient deprivation due to the limited diet). We did not evaluate changes in the gut microbial community under starvation conditions and additional studies of such conditions are warranted.

### 3.6. Significance of the ubiquity of PS degradation by mealworms

The results of the global survey strongly suggest that mealworm ingestion of plastic foams, like Styrofoam, is genetically-programmed and ubiquitous. Rapid PS depolymerization was observed, as observed previously (Yang et al., 2015a; Yang et al., 2018), and this capability is apparently conserved in mealworm populations throughout the world. These mealworm populations ingest and use PS foam as a food (energy) source for survival, and this behavior is not limited to PS: PE can be consumed even more rapidly than PS. Approximately  $49.0 \pm 1.4\%$  of ingested PE was converted into a putative gas fraction (i.e. CO<sub>2</sub>) (Brandon et al., 2018). Evidence of mealworm consumption is now available for many other materials, including PP, PVC, PET and PUR at laboratories in the USA and China (data not shown). Other academic researchers have reported mealworm consumption of PVC and PLA (Božek et al., 2017).

Chewing of plastic into small fragments increases the surface area of the ingested

plastic and thus likely enhances enzymatic depolymerization, but depolymerization of the ingested plastic was not completed in the 14-18 hour transit time through the mealworm gut. Residual PS in the frass contributes to the THF extractable fraction, constituting ~35% of the weight of frass (Yang et al., 2018). Further testing is needed to determine whether this residual is recalcitrant, say, for example, because of crystallinity differences in the PS feedstock, or whether this PS could be degraded with additional residence time in the mealworm gut. In any case, this work establishes that ingestion of PS and PE plastic foam can provide energy for maintenance of mealworm populations for 4-5 weeks. However, a sustained diet of plastic without supplemental nutrition does not enable reproduction of mealworms (Yang et al., 2018) and eventually leads to cannibalism.

The ability of insects to depolymerize diverse plastics is likely ubiquitous and not restricted to mealworms. The fact that insect pests, including darkling beetles (*Tribolium castaneum*, *Rhizopertha* *Lasioderma serricorne*, *Tenebrioide mauretanicus*, *Zophobas morio* etc. in family *Tenebrionidae* ) and several moths and their larvae (*Plodia interpunctella*, *Galleria mellonella* in the Family *Pyrallidae*) can chew, eat, and penetrate various plastic packing materials has been established since the 1950s (Gerhardt and Lindgren, 1954; Cline, 1978; Newton, 1988; Bombelli et al., 2017). These early studies established that insect larvae chew and damage PE, PVC and polyester films regardless of whether the film was used to cover food (Cline 1978), suggesting that the larvae of insect pests may also ingest plastics as food. Recent studies indicated that the larvae of *Plodia interpunctella* (Indian meal moth) and *Galleria mellonella*, or honey waxworms, can chew and likely degrade PE films (Yang et al., 2014; Bombelli et al., 2017).

#### 4. Conclusions

This work establishes that mealworms have an intrinsic capacity for biodegradation of PS due to their intrinsic eating and chewing behaviors and gut microbe-dependent oxidative digestive machinery. Digestion of PS contribute to the energy needs of the mealworms. These features appear to be ubiquitous within mealworms from diverse geographic locations and can be enhanced by co-feeding of nutrient sources such as bran. The contributing gut microbial communities are diverse and subject to dietary control.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at ...

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**Fig. 1.** Locations where mealworm consumption of plastic has been observed (green) and where mealworms have been tested in biodegradation studies (red dots). The figure was adapted from [www.paonazario.com](http://www.paonazario.com).

**Fig. 2.** Plastic feeding behavior of mealworms used for PS biodegradation tests. Sources 1 to 5 are from the USA, source 6 is from the UK, and sources 7-12 are from China. See also Table 1.

**Fig. 3.** Changes in molecular weights  $M_w$  (blue) and  $M_n$  (yellow) and in molecular weight distribution (MWD) of PS feedstock (black curves) and PS polymer residues extracted from frass samples (red curves) for mealworms from each of the 12 sources. **Panels 1-5** summarize data for sources in the USA; **Panel 6** illustrates data for Northern Ireland; and **Panels 7-12** summarize data for Chinese sources.

**Fig. 4.** Formation of functional intermediate groups of PS degradation and the effect of gentamicin inhibition on Styrofoam depolymerization. **Panel A** shows FTIR spectra of PS feedstock (Control), and frass of mealworms (Source 1) fed with PS only, PS plus bran, and bran only. **Panel B** shows FTIR spectra of frass of mealworms (Source 2) fed with PS plus bran, and bran only. **Panel C** shows the decrease in gut bacterial counts within mealworms after feeding of gentamycin over a 7day period. **Panel C** shows  $M_n$  and **Panel D** shows  $M_w$  for the PS feedstock (blue) compared to values for the frass from mealworms receiving gentamycin (orange) and without gentamycin (green) from USA sources 1-5

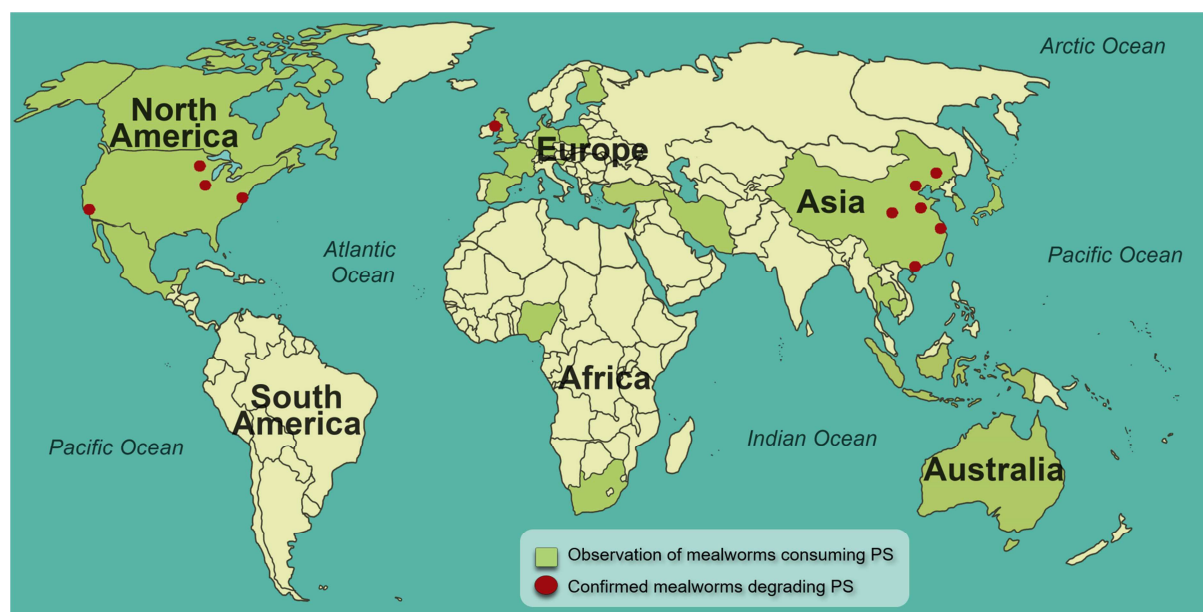
**Fig. 5** Relative abundances of dominant (A) phyla, (B) families, and (C) genera in internal microbial communities from original mealworms (orange) versus those fed with

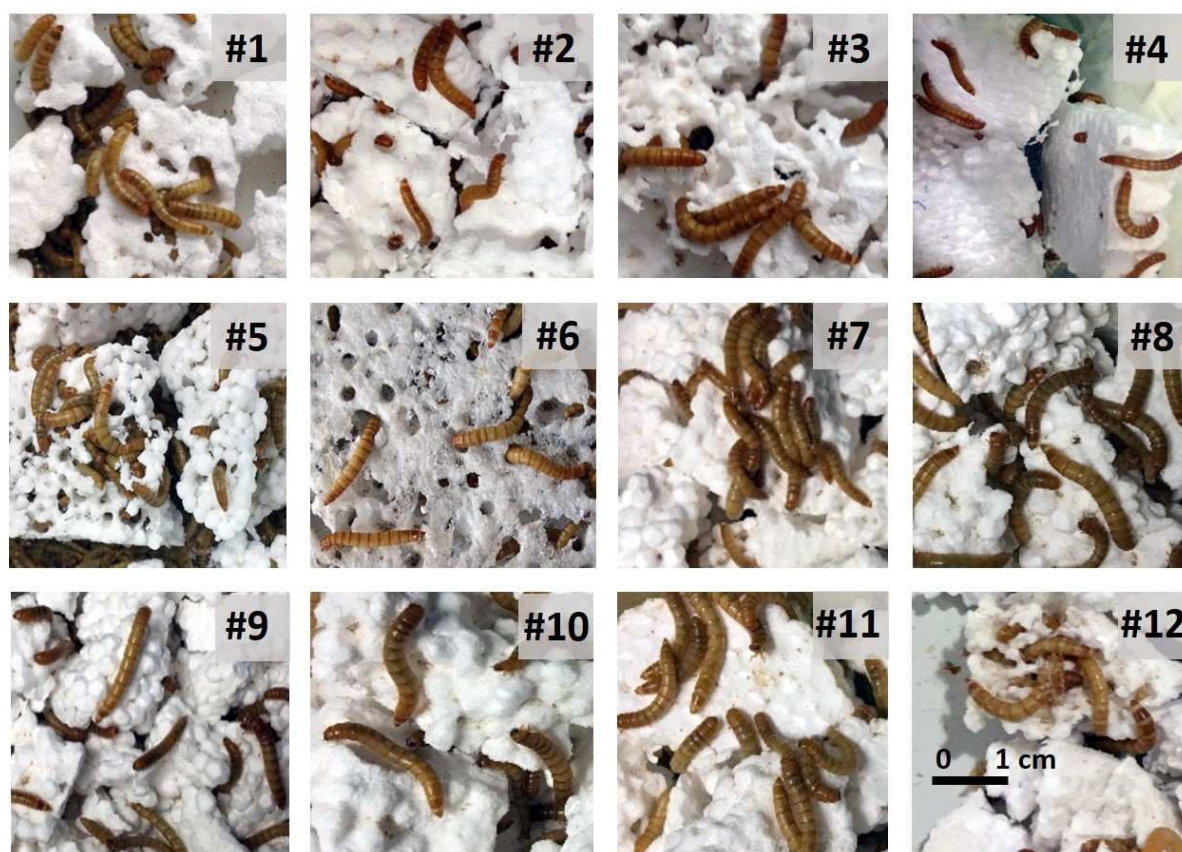
PS (gold) and bran plus PS (purple). Error bars indicate standard deviations for multiple mealworm samples. Taxa with a relative abundance less than the threshold stated in each figure label are not shown. Significance is shown with lowercase letters; all other comparisons were not significantly different after adjusting for multiple comparisons.

**Table 1.** Summary of PS biodegradation studies conducted at Stanford, Belfast and Beijing.

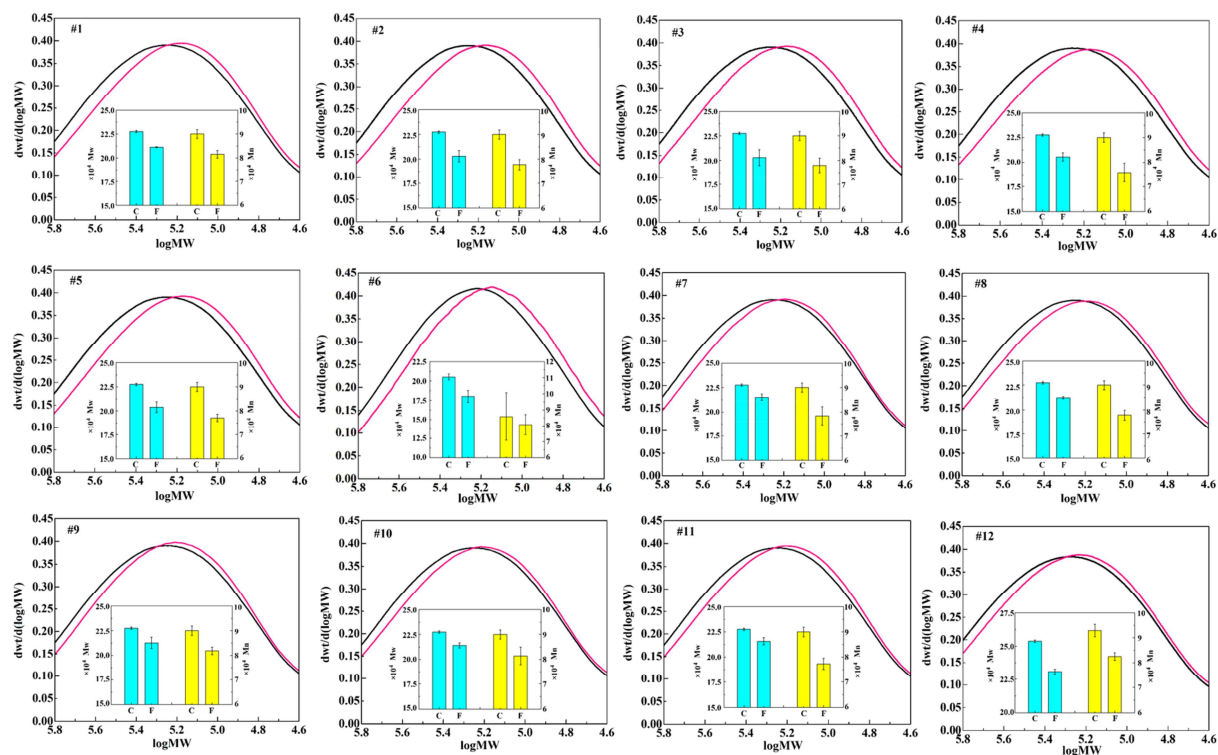
Mealworm source (no. mealworms)	Initial wt mg worm <sup>-1</sup>	Feed	Final day	Final wt mg worm <sup>-1</sup>	Survival rate %	Specific PS consumption rate*	
						mg PS 100 worms <sup>-1</sup> d <sup>-1</sup>	mg PS g worms <sup>-1</sup> d <sup>-1</sup>
1 USA (120)	76.54±1.55	PS+B	32	78.64±0.42	89.2±0.8	40.67±0.95	5.31±0.12
		PS	32	74.77±0.36	81.7±0.8	18.74±0.31	2.44±0.04
2 USA (120)	73.98±0.81	PS+B	32	74.17±1.94	90.8±3.3	45.59±0.58	6.16±0.07
		PS	32	71.76±0.35	88.3±0.8	20.71±0.51	2.80±0.07
		B	32	nd	90.8±1.7	nd	nd
		Unfed	32	nd	74.2±4.2	nd	nd
3 USA (120)	91.16±0.76	PS+B	32	137.90±1.02	88.3±0.8	29.36±1.90	2.56±0.10
		PS	32	94.27±0.84	87.5±1.7	14.15±0.35	1.55±0.03
		B	32	nd	87.5±1.7	nd	nd
		Unfed	32	nd	70.8±5.0	nd	nd
4 USA (120)	46.34±0.89	PS+B	32	48.14±0.91	90.8±2.5	19.24±1.17	4.15±0.25
		PS	32	47.88±0.79	90.8±1.7	8.46±0.14	1.83±0.03
		B	32	nd	89.2±0.8	nd	nd
		Unfed	32	nd	75.8±2.5	nd	nd
5 USA (120)	84.09±1.27	PS+B	32	86.18±2.16	84.2±1.7	35.97±0.05	4.28±0.01
		PS	32	82.18±1.01	82.5±1.7	16.46±0.28	1.96±0.33
		B	32	nd	85.0±2.5	nd	nd
		Unfed	32	nd	69.2±3.3	nd	nd
6 Northern Ireland (200)	33.54±0.59	PS+B	35	40.98±0.06	98.0±0.0	25.82±0.17	7.64±0.05
		PS	35	35.99±2.90	95.5±1.0	19.21±0.21	5.92±0.06
		Unfed	35	30.80±1.07	89.0±2.0	nd	nd
		PS+B	98	49.09±1.84	81.5±7.1	nd	nd
		PS	98	51.60±15.92	11.5±4.9	nd	nd
		Unfed	98	52.28±3.54	11.8±1.1	nd	nd
7 China (500)	63.25	PS+B	24	117.84	92.7	45.32	7.17
8 China (500)	76.03	PS+B	24	104.39	89.2	31.34	4.12
9 China (500)	80.23	PS+B	24	132.74	92.3	24.39	3.04
10 China (500)	61.04	PS+B	24	111.97	90.5	29.45	4.90
11 China (500)	85.05	PS+B	24	125.13	95.3	41.24	4.85
12 China (500)	51.95	PS+B	24	62.92	92.8	20.36	3.92

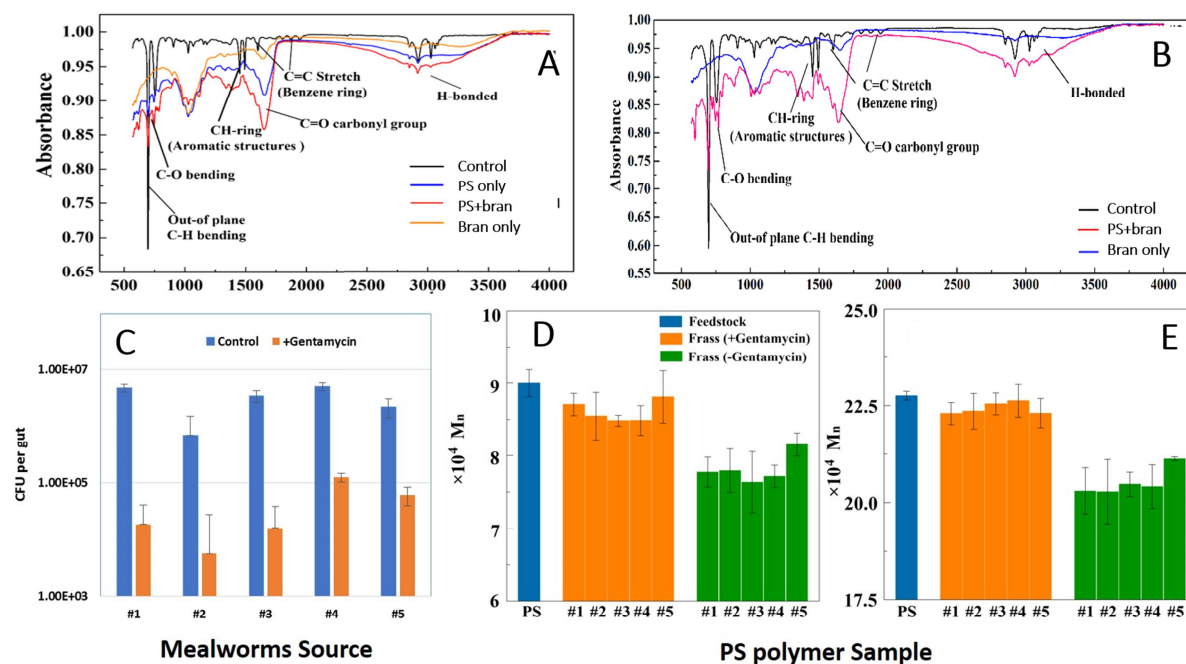
\*Specific PS consumption rates were computed as the mass of PS consumed over the test period (32 days for USA sources, 35 days for the UK source, and 24 days for Chinese sources) divided by the initial number of mealworms (column 7) or by the average initial weight of mealworms (column 8). PS=polystyrene; B=bran; nd = not determined.

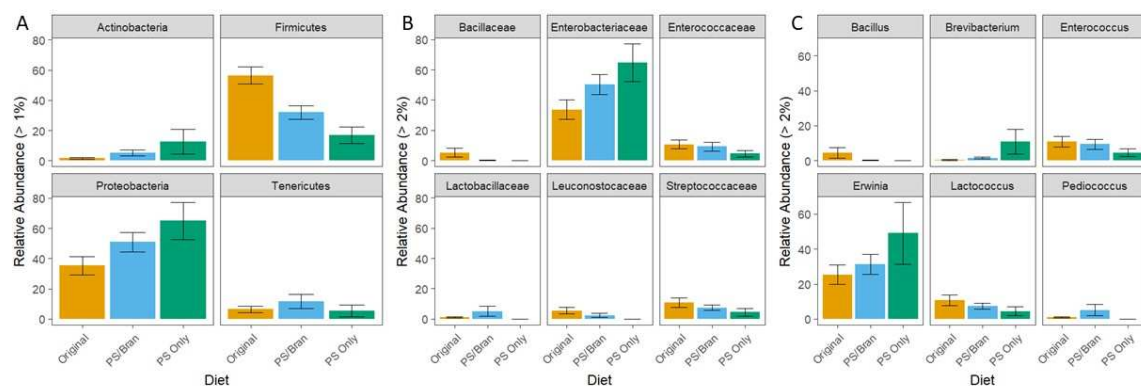












**HIGHLIGHTS**

- Consumption of polystyrene (PS) by mealworms observed in 22 countries
- PS degrades in mealworms obtained from 12 locations in the USA, UK and China
- Addition of nutrition enhances survival rate and PS consumption rate
- Antibiotics depresses gut microbes and severely inhibits PS degradation
- PS feeding shifts mealworm gut microbiome